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Essential Roles of Hydrophobic Residues in Both MD-2 and Toll-like Receptor 4 in Activation by Endotoxin*

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Gram-negative bacterial endotoxin (*i.e.* lipopolysaccharide (LPS)) is one of the most potent stimulants of the innate immune system, recognized by the TLR4·MD-2 complex. Direct binding to MD-2 of LPS and LPS analogues that act as TLR4 agonists or antagonists is well established, but the role of MD-2 and TLR4 in receptor activation is much less clear. We have identified residues within the hairpin of MD-2 between strands five and six that, although not contacting acyl chains of tetraacylated lipid IVa (a TLR4 antagonist), influence activation of TLR4 by hexaacylated lipid A. We show that hydrophobic residues at positions 82, 85, and 87 of MD-2 are essential both for transfer of endotoxin from CD14 to monomeric MD-2 and for TLR4 activation. We also identified a pair of conserved hydrophobic residues (Phe-440 and Phe-463) in leucine-rich repeats 16 and 17 of the TLR4 ectodomain, which are essential for activation of TLR4 by LPS. F440A or F463A mutants of TLR4 were inactive, whereas the F440W mutant retained full activity. Charge reversal of neighboring cationic groups in the TLR4 ectodomain (Lys-388 and Lys-435), in contrast, did not affect cell activation. Our mutagenesis studies are consistent with a molecular model in which Val-82, Met-85, and Leu-87 in MD-2 and distal portions of a secondary acyl chain of hexaacylated lipid A that do not fit into the hydrophobic binding pocket of MD-2 form a hydrophobic surface that interacts with Phe-440 and Phe-463 on a neighboring TLR4·MD-2·LPS complex, driving TLR4 activation.

Bacterial lipopolysaccharide (LPS)[3] is recognized by the innate immune system of vertebrates via an elaborate mechanism involving the membrane receptor TLR4 (1, 2). The extracellular (or cell surface) proteins LPS-binding protein and CD14 promote extraction and transfer of individual molecules of LPS from the Gram-negative bacterial outer membrane to MD-2, either secreted monomeric soluble (s)MD-2 or MD-2 bound with high affinity to the ectodomain of TLR4 (3–7). In contrast to other Toll-like receptors, TLR4 requires an additional molecule, MD-2, for ligand recognition (8). In contrast to MD-2, there has been no evidence of direct binding of LPS to TLR4 (9, 10). Although LPS, and particularly the lipid A portion of LPS, is generally conserved among Gram-negative bacteria, there are many variables in LPS structure that affect TLR4 activation. Most important is the acylation pattern of the lipid A moiety, which represents the minimal segment of LPS that can trigger activation of TLR4 (11). Comparison of crystal structures of MD-2 with and without bound tetraacylated lipid IVa indicates no significant alteration of the protein fold in the absence or presence of bound ligand (12). It has been proposed that both LPS and MD-2 are key to the different effects of tetra-*versus* hexaacylated LPS on TLR4 (8, 13, 14). Lipid IVa complexed to murine MD-2 has weak agonist effects on murine TLR4 but acts as a receptor antagonist in the same complex containing human MD-2. Hexaacylated endotoxins complexed to human or murine MD-2 act as potent TLR4 agonists. The crystal structure of the TLR4·MD-2·eritoran complex revealed that MD-2 binds to the N-terminal region of TLR4 (15). It seems likely that for TLR4 activation, there needs to be an additional interaction between two ternary TLR4·MD-2·LPS complexes, which is agonist-dependent (15–17). Because tetraacylated and hexaacylated endotoxins that act, respectively, as TLR4 antagonists and agonists differ only in their acylation pattern, we speculated that hydrophobic protein-lipid A interactions are essential in the agonist properties of hexaacylated lipid A. To pursue this hypothesis, we used molecular modeling to select and test the involvement of solvent-exposed hydrophobic residues of MD-2 and TLR4, which we reasoned could be needed for TLR4 activation. We show by mutagenesis studies that residues on the solvent-exposed hairpin of MD-2 support transfer of endotoxin from CD14 to MD-2 and TLR4 activation only when these sites contain hydrophobic residues. In the ectodomain of TLR4, we have identified two neighboring phenylalanine residues located on the convex face of consecutive leucine rich repeats that are required for LPS-triggered TLR4 activation. From those results and molecular docking, we propose that amino acid side chains of both MD-2 and TLR4 ectodomain form an acyl chain binding site, which envelops part of an acyl chain of lipid A that cannot fit into the binding pocket of MD-2 in a TLR4·MD-2 complex and represents a key to LPS-induced TLR4 activation.

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[3] The abbreviations used are: LPS, lipopolysaccharide; LOS, lipooligosaccharide; HEK, human embryonic kidney; wt, wild-type; TLR, Toll-like receptor.

MATERIALS AND METHODS

Cell Culture and Reagents—The human embryonic kidney (HEK) 293 cells were provided by Dr. J. Chow (Eisai Research Institute, Andover, MA). HEK293 cells stably transfected with TLR4 (HEK293/TLR4#BF1) were provided by Dr. Douglas Golenbock (University of Massachusetts Medical Center) and Dr. Andra Schromm (Research Center Borstel). S-LPS (from *Salmonella abortus equi*) was purchased from Sigma. *Escherichia coli*-type lipid A (compound 506) was obtained from the Peptide Institute (Osaka, Japan). Purified [^{3}H]lipooligosaccharide (LOS) (25,000 cpm/pmol) was isolated from an acetate auxotroph of *Neisseria meningitidis* serogroup B after metabolic labeling, as described (18). Sephacryl S200 HR size exclusion gel was purchased from GE Healthcare. Human serum albumin was obtained as an endotoxin-free, 25% stock solution (Baxter Health Care, Glendale, CA). Anti-tetra-His antibodies and goat anti-mouse horseradish peroxidase-conjugated secondary antibodies were from Qiagen (Valencia, CA) and Jackson Immunologicals (West Grove, PA), respectively. LPS-binding protein and sCD14 were gifts from XOMA (Berkeley, CA) and Amgen Corp. (Thousand Oaks, CA), respectively.

Site-directed Mutagenesis—All mutations were introduced into pEFBOS-hMD-2-FLAG-His plasmid for MD-2 mutants or pCMV-TLR4-FLAG plasmid for TLR4 mutants using a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. All plasmids were sequenced to confirm the mutation. Primer sequences will be made available upon request.

Preparation of [^{3}H]LOS$_{agg}$ and [^{3}H]LOS·sCD14 Complex—[^{3}H]LOS$_{agg}$ and [^{3}H]LOS·sCD14 complex were prepared as previously described (4, 18) Briefly, [^{3}H]LOS$_{agg}$ ($M_r > 20 \times 10^6$) were obtained after hot phenol extraction of [^{3}H]LOS from metabolically labeled bacteria followed by ethanol precipitation of [^{3}H]LOS$_{agg}$ and ultracentrifugation. Monomeric [^{3}H]LOS·CD14 complexes ($M_r \sim 60{,}000$) were prepared by treatment of [^{3}H]LOS$_{agg}$ for 30 min at 37 °C with a substoichiometric LPS-binding protein (molar ratio, 100:1 LOS:LPS-binding protein) and 1–1.5× molar excess of sCD14 followed by gel exclusion chromatography (Sephacryl S200, 1.6 × 70-cm column) in phosphate-buffered saline, pH 7.4, 0.03% human serum albumin to isolate monomeric [^{3}H]LOS·sCD14 complex. Radiochemical purity of [^{3}H]LOS$_{agg}$ and [^{3}H]LOS·sCD14 was confirmed by Sephacryl S500 (LOS$_{agg}$) or S200 ([^{3}H]LOS·sCD14) chromatography (4, 18).

Production and Reaction of Secreted MD-2 and sMD-2/TLR4 Ectodomain (TLR4$_{ecd}$) with [^{3}H]LOS·sCD14 Complex—HEK293T cells were plated in a 6-well plate with 10% fetal bovine serum in Dulbecco's modified Eagle's medium. Cells were transfected the following day with an expression plasmid encoding wild-type (wt) or mutant MD-2 (±wt TLR4$_{ecd}$; residues 24–631) using PolyFect reagent (Qiagen) as previously described (9). After 12–16 h, the medium was changed with 1.5 ml of serum-free medium (Dulbecco's modified Eagle's medium, Invitrogen) + 0.1% human serum albumin. The medium was spiked with [^{3}H]LOS·sCD14 (1 nM) at the time of medium replacement to permit reaction of [^{3}H]LOS·sCD14 with MD-2 upon secretion. Reaction products were analyzed by Sephacryl HR S200 (1.6 × 30 cm) chromatography in phosphate-buffered saline. Fractions (0.5 ml) were collected at a flow rate of 0.5 ml/min at room temperature using AKTA Purifier or Explorer 100 fast protein liquid chromatography (GE Healthcare). Radioactivity in collected fractions was analyzed by liquid scintillation spectroscopy (Beckman LS liquid scintillation counter). Recoveries of [^{3}H]LOS were ≥70% in all cases. All solutions used were pyrogen-free and sterile-filtered.

HEK293 Cell Activation Assay - Luciferase Reporter Assay—For testing the activity of MD-2 mutants, HEK293#BF1 hTLR4 cells were seeded at 7×10^4 cells/well in 96-well Costar plates (Corning, NY). For testing the activity of TLR4 mutants, HEK293 cells were seeded at 5×10^4 cells per well. Cells were incubated overnight in a humidified atmosphere (5% CO_2) at 37 °C. The next morning cells were cotransfected for 4 h with pEFBOS-hMD-2-FLAG-His and/or pCMV-TLR4-FLAG (only when using HEK293 cells) as well as NF-κB-dependent luciferase and constitutive Renilla reporter plasmids using Lipofectamine 2000 (Invitrogen). After 4 h, the medium was removed and replaced with Dulbecco's modified Eagle's medium + 10% fetal bovine serum. The following day the cells were incubated with LPS for 16 h (see the individual figures for the doses of LPS tested). Cells were lysed in 1× reporter assay lysis buffer (Promega) and analyzed for reporter gene activities using a dual-luciferase reporter assay system on a Mithras LB940 luminometer. Relative luciferase activity was calculated by normalizing each sample's luciferase activity for constitutive Renilla activity measured within the same sample.

To assess the activity of wt and mutant MD-2 secreted in the absence of TLR4, HEK293 cells were transfected with pEFBOS-hMD-2-FLAG-His, and HEK293#BF1 hTLR4 cells were cotransfected with NF-κB-dependent luciferase and constitutive Renilla reporter plasmids. Aliquots of the conditioned medium of HEK293 cells containing sMD-2 were added to the HEK293#BF1 hTLR4 cells, which were then incubated with LPS + 2% serum. Cell activation was analyzed as described above.

Immunoblotting—To detect polyhistidine-labeled wt and mutant MD-2, an anti-polyhistidine antibody (Tetra-His antibody, Qiagen) was used. Aliquots of conditioned medium from transfected and mock-transfected HEK293 cells (see above) were harvested after 24 h. Equal volumes of the medium and Laemmli sample buffer containing dithiothreitol were combined, and each sample was electrophoresed (Bio-Rad mini gel system) through a 4–15% gradient acrylamide gel (Tris/HEPES/SDS buffer) and transferred to nitrocellulose membrane. The membrane was washed with Tris-buffered saline, pH 7.5, containing 0.05% Tween 20 and 0.2% Triton X-100 (TBSTT), blocked to reduce nonspecific background with 5% dried nonfat milk in TBSTT for 1 h at 25 °C, and incubated with the anti-His$_4$ antibody in the blocking solution overnight. After washing with TBSTT, the blot was incubated with goat anti-mouse IgG conjugated to horseradish peroxidase for 1 h at 25 °C in the blocking solution and washed extensively with TBSTT. Blots were developed using the Pierce SuperSignal substrate system. By reducing immunoblot samples, each MD-2 species was converted to the monomeric form (19).

Surface Expression of TLR4 Mutants—HEK293T cells were seeded in a 6-well plate (1×10^6 cells per well) and transfected the following day with plasmids encoding wt or mutant TLR4. After 48 h the cells were harvested and washed twice with phosphate-buffered saline. Cells were resuspended with 100 μl of fluorescence-activated cell sorter buffer containing 8 μg/ml rabbit anti-FLAG and incubated on ice for 20 min. After this incubation the cells were washed twice with fluorescence-activated cell sorter buffer, resuspended, and incubated for 20 min in the dark with 2 μg/ml DyeMer 488/615 goat anti-rabbit IgG. The cells were then washed twice more with fluorescence-activated cell sorter buffer and resuspended in 500 μl of phosphate-buffered saline. Flow cytometry analysis was performed on EPICS ALTRA flow cytometer (Beckman Coulter). In each sample 10,000 cells were analyzed. Collected data were analyzed by using WinMDI flow cytometry application.

Molecular Docking—The structural models of the complex were calculated using the program HADDOCK (20, 21) that has been implemented in CNS (22, 23) for structure calculations and makes use of python scripts derived from ARIA (24) for automation. HADDOCK (High Ambiguity Driven protein-protein Docking) makes use of biophysical interaction data such as chemical shift perturbation data resulting from NMR titration experiments, mutagenesis data, or bioinformatic predictions that are introduced as ambiguous interaction restraints to drive the docking process. In our application we used mutagenesis data from this report to obtain a model of the activating (TLR4·MD-2)′ -(TLR4·MD-2)′′ heterodimer. The TLR4·MD-2 complex was constructed by splicing the x-ray structures of human TLR4-TV3 hybrid complexed with MD-2 and the LPS antagonist Eritoran (PDB code 2Z65) and VT3 hybrid between TLR4 and hagfish variable lymphocyte receptor (PDB code 2Z66). Two of these TLR4·MD-2 units, ′ and ′′, were used in the docking calculations that were driven by the following ambiguous interaction restraints; residues 82, 85, 87, and 126 of MD-2 should be close to any or all of the residues 440 and 463 of TLR4′′, and the C-terminal residues of TLR4′ ectodomain should not be far from the C terminus of the TLR4′′ ectodomain. Twenty different rigid docking calculations of the heterodimer followed by refinement of the 10 best structures (in terms of docking interaction) with selected flexible side chains were performed. The structure with the most favorable docking interaction was used for constructing a final model.

RESULTS

Selection of Hydrophobic Mutations in the Hairpin Loop of MD-2—Structure-activity relationships in MD-2 have been extensively investigated by point mutations. These studies enabled the prediction of TLR4 and LPS binding sites in MD-2 before the structure of the TLR4·MD-2·LPS complex was determined (6, 25). Crystal structures of MD-2 did not show major structural changes in MD-2 whether or not MD-2 contained bound tetraacylated lipid IVa or was bound to the TLR4 ectodomain ($TLR4_{ecd}$). However, comparison of the structures of MD-2·lipid IVa and MD-2·eritoran complexes reveal discrete conformational changes induced by binding eritoran in the loop containing Phe-126 and the hairpin loop containing residues 82–87 (Fig. 1). As a result the complex with bound eritoran has a narrower opening to the hydrophobic pocket and closer proximity of the loop containing residues 82–87 to Phe-126 (17, 26). Replacement of Phe-126 with alanine does not affect binding of hexaacylated LPS/LOS but prevents TLR4 activation (26). Numerous studies have shown that the acylation pattern of lipid A (*e.g.* number of fatty acids, length of the acyl chains, and their arrangement) also governs TLR4 activation when wt MD-2 and TLR4 are expressed. Crystal structures of MD-2 complexed with tetraacylated lipid A analogues indicate that the hydrophobic pocket of MD-2 can accommodate four, potentially five acyl chains. In eritoran, a tetraacylated analogue of lipid IVa, one of the four acyl chains is a long unsaturated acyl chain at position R2′ that is bent due to its *cis* double bond (Fig. 1) and, thus, occupies a similar position as would a fifth acyl chain in pentaacylated endotoxin (15). However, the crystal structures of these complexes suggest that a major expansion of the binding pocket of MD-2 would be required to accommodate the six fatty acyl chains typically present in LPS (lipid A) species that are potent TLR4 agonists. Alternatively, if the binding pocket of MD-2 did not change, parts of the sixth fatty acyl chain could be accommodated by residues on the surface of MD-2, in close proximity to the hydrophobic binding pocket. Based on these MD-2 models, we selected the hairpin

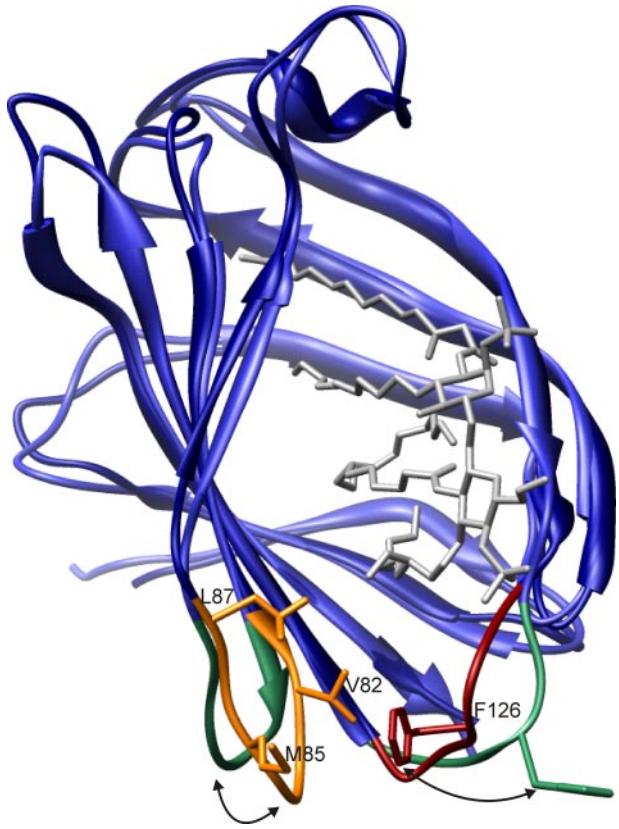

FIGURE 1. **Overlay of ribbon diagrams of crystal structures of MD-2 complexed to lipid IVa (2E59) or eritoran (2Z65) reveals localized structural variation.** For clarity, only bound eritoran is shown. The MD-2 structures in these two complexes are highly similar except in the hairpin loop between amino acid residues 82–87 and in the loop around Phe-126, which shift in the structure with bound eritoran (shown as a *stick* representation in *gray*) toward each other and formation of a hydrophobic surface leading to the large hydrophobic pocket of MD-2. Side chains of amino acid residues investigated by mutations are shown: Val-82, Met-85, Leu-87, and Phe-126.

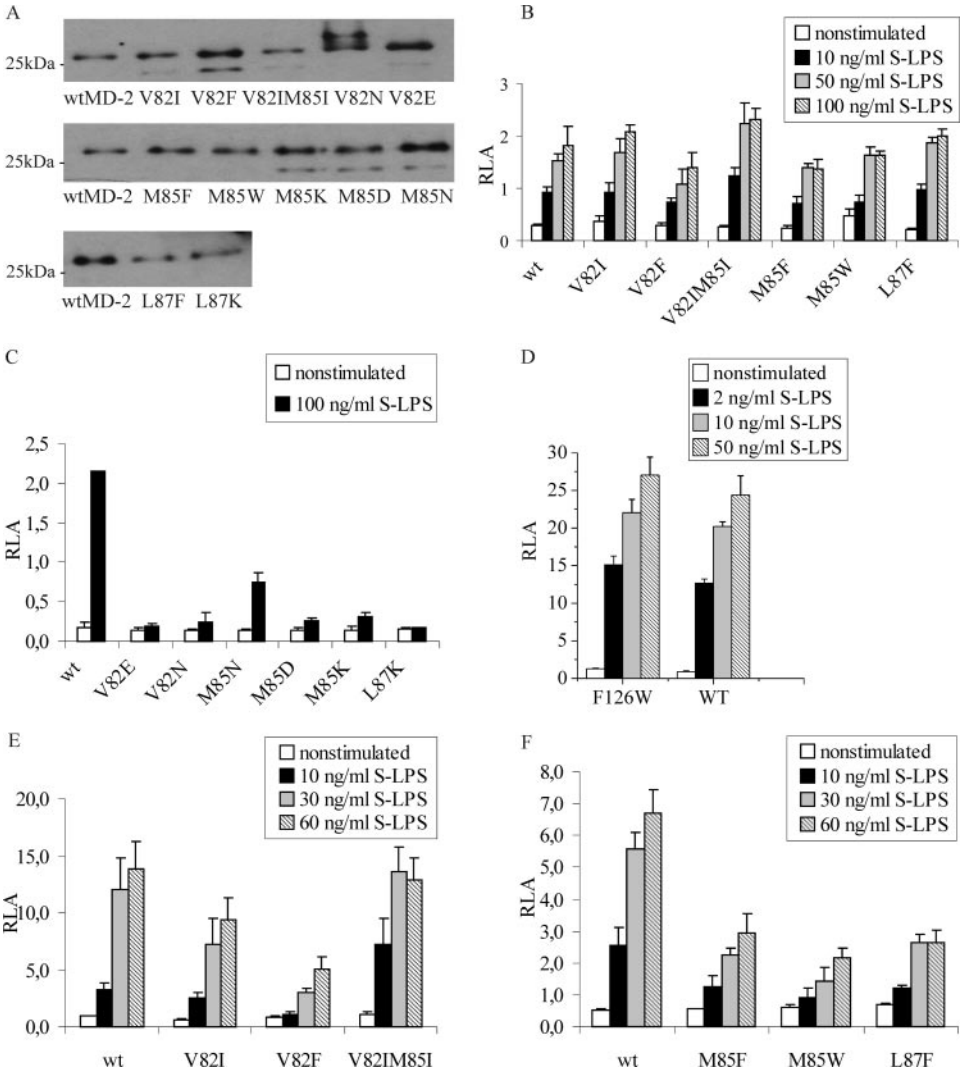

FIGURE 2. **Effect of mutations of MD-2 at residues 82, 85, 87, and/or 126.** *A*, secretion of MD-2 mutants into the medium. HEK293T cells were transfected with expression plasmids encoding wt or mutant MD-2. Conditioned medium with secreted MD-2 was analyzed by SDS-PAGE/immunoblot as described under "Materials and Methods." Multiple bands reflect differences in glycosylation; the V82N mutation created a new glycosylation site. *B*, cell activation by MD-2 replacement of residues at positions 82, 85, and 87 with hydrophobic residues. *C*, cell activation by MD-2 mutants with residues at position 82, 85, or 87 replaced with polar residues. *D*, replacement of Phe-126 with tryptophan residue. *E* and *F*, effect of mutations on the biological activity of soluble secreted MD-2. HEK293 cells were transfected with expression plasmids encoding wt or mutant sMD-2. Cell culture media containing sMD-2 were collected and transferred to HEK293#BF1 cells expressing TLR4, which had been transfected with reporter luciferase plasmids. HEK293#BF1 cells were then stimulated with LPS and assayed for luciferase activity. Results shown are from one experiment in triplicate and are representative of at least three independent experiments. *RLA*, relative luciferase activity, represents luciferase activity divided by *Renilla* activity.

loop in the region 82–87 between β strands 5 and 6 for a detailed investigation, as it contains several conserved hydrophobic residues and is near the lipid IVa binding pocket. We prepared hydrophobic, polar, and charged mutations at positions Val-82, Met-85, and Leu-87. All of the side chains are significantly exposed to solvent and contribute to the hydrophobicity of MD-2. None of the selected residues is in contact with atoms of the antagonistic lipid IVa or eritoran in the crystal structure but are positioned so that they tile the surface of the hairpin, which could come in contact with portions of a fatty acid(s) of the agonistic hexaacylated lipid A that cannot be accommodated within the pocket of MD-2.

Effect of Mutations in the Loop Comprising Residues 82–87 of MD-2 on TLR4 Activation—All the MD-2 mutants were expressed at similar levels as the wild type MD-2 (Fig. 2*A*). Single mutations making conservative substitutions of hydrophobic residues at positions 82, 85, and 87 (*e.g.* V82I) had little or no effect on MD-2 functional activity as assessed by the ability of MD-2 to support LPS-triggered TLR4 activation when coexpressed with TLR4 (Fig. 2*B*) or expressed and secreted without TLR4 and added to cells expressing TLR4 alone (Fig. 2, *E* and *F*). A double mutant V82IM85I reproducibly showed slightly increased MD-2 activity in comparison to wt MD-2 (Fig. 2, *B* and *E*). Substitution with aromatic amino acids (*e.g.* V82F, M85F, M85W) containing more bulky, hydrophobic side chains caused a modest diminution of MD-2 functional activity that was somewhat greater when these mutants were expressed and secreted without TLR4 (Fig. 2, *F versus B*; see "Discussion"). In contrast, all substitutions at these sites with polar or charged residues (V82N, V82E, M85N, M85K, M85D, L87K) showed a marked decrease in LPS-triggered cell activation (Fig. 2*C* and Table 1). In the case of Met-85, which is more exposed to solvent than Val-82 or Leu-87, replacement with asparagine impaired MD-2 function slightly less than replacement with a charged residue.

Effect of Mutations in the Loop Comprising Residues 82–87 of MD-2 on LPS Binding—Mutations of Val-82, Met-85, or Leu-87 that reduce MD-2 function could do so by affecting endotoxin binding. To test this possibility more directly, we monitored the reaction of added radiolabeled monomeric LOS·sCD14 with wt or mutant sMD-2 that was expressed and secreted by transiently transfected HEK293T cells. Transfer of [^{3}H]LOS from CD14 (M_r ~60,000) to sMD-2 (M_r ~25,000) was monitored by size exclusion chromatography, as previously described (4). MD-2 mutants that retained the ability of wt MD-2 to support LPS-triggered TLR4 activation (V82I, L87F, and V82IM85I; see above) also retained the ability of wt MD-2 to react with [^{3}H]LOS·sCD14 and form monomeric [^{3}H]LOS.MD-2 (Fig. 3*B*). Substitutions of aromatic amino acids at Val-82 or Met-85 that caused a modest

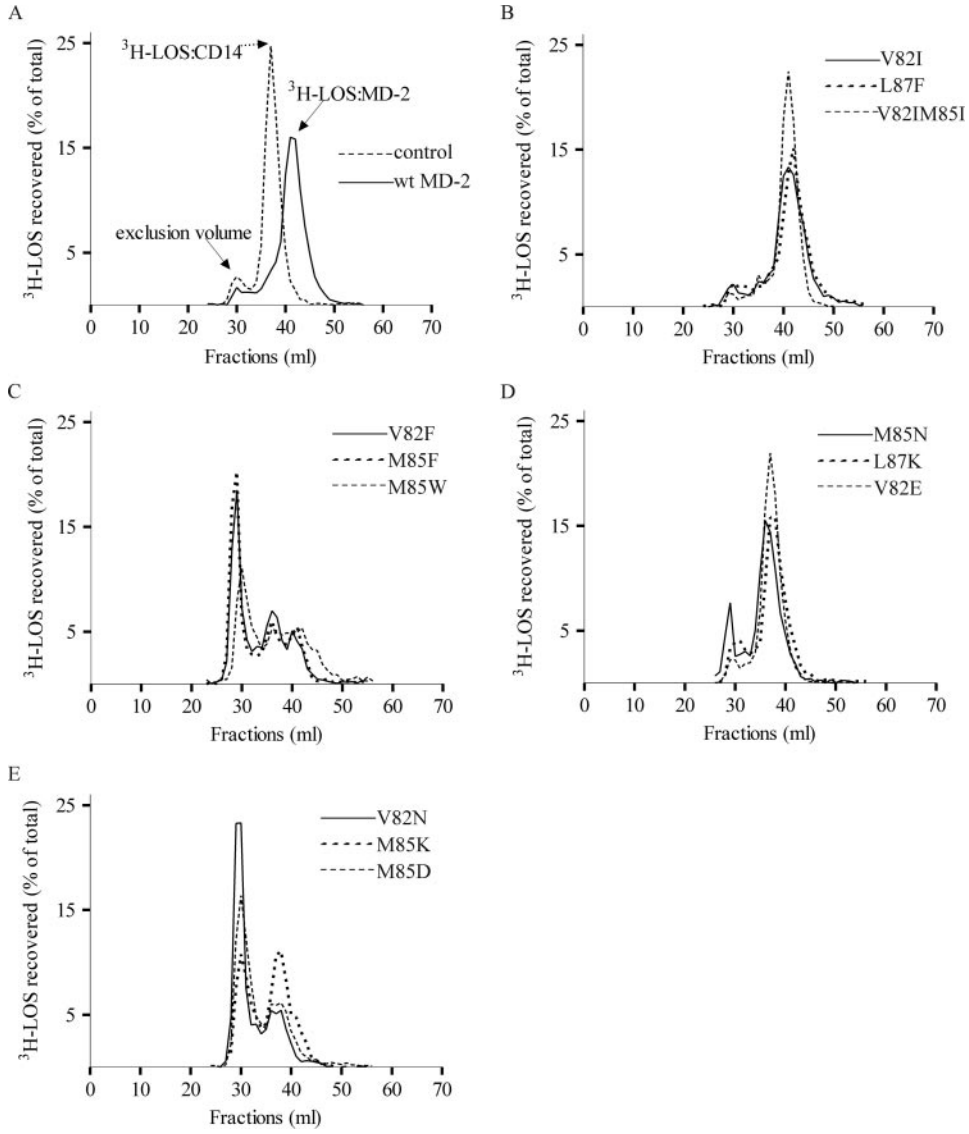

FIGURE 3. **Effect of differences in polarity of amino acids at residues 82, 85, and/or 87 of MD-2 on the ability of sMD-2 to react with [^{3}H]LOS·sCD14 to form monomeric [^{3}H]LOS·MD-2.** wt and mutant sMD-2 were produced using transiently transfected HEK293T cells and tested for the ability to bind LOS (*i.e.* transfer LOS from the [^{3}H]LOS·CD14 complex) using Sephacryl S200 chromatography as described under "Materials and Methods." ^{3}H-Labeled LOS in collected fractions was detected by liquid scintillation spectroscopy. Total recovery of [^{3}H]LOS was typically >70%. Note that the peaks of elution of [^{3}H]LOS·sCD14 and [^{3}H]LOS·MD-2 were at 37 and 42 ml, respectively. The results shown are from one experiment, representative of at least two independent determinations.

TABLE 1

Summary of biochemical properties of MD-2 mutants at positions 82, 85, and 87

agg, formation of large aggregates with LOS; ND, not determined; +, same as wild type; ++, increased; (+), impaired; (−), little; −, no binding/activity.

	MD-2 mutants	Cell activation	LOS transfer from CD14	LOS transfer to the complex with ecdTLR4
Hydrophobic mutations	V82I	+	+	ND
	L87F	+	+	ND
	V82IM85I	++	+	ND
	V82F	(+)	(+); agg	+
	M85F	(+)	(+); agg	+
	M85W	(+)	(+); agg	+
Polar mutations	V82N	−	agg	agg
	M85D	−	agg	agg
	M85K	−	agg	−
	M85N	(−)	agg	(+)
	V82E	−	−	−
	L87K	−	−	−

diminution of MD-2 functional activity (V82F, M85F, M85W; see above) also caused reduced generation of monomeric [^{3}H]LOS.MD-2, with increased conversion of [^{3}H]LOS to a more aggregated state (Fig. 3*C*; see "Discussion"). None of the polar mutants (V82N, V82E, M85D, M85K, M85N, L87K) reacted with [^{3}H]LOS·sCD14 to form monomeric [^{3}H]LOS·MD-2 (Fig. 3, *D* and *E*), matching their markedly diminished ability to support TLR4 activation by LPS (Fig. 2*C*).

LPS Binding to sMD-2 Coexpressed with the TLR4 Ectodomain—We have previously shown that the TLR4 ectodomain can rescue the functional activity (*i.e.* LPS binding) of MD-2 variants that are prone to aggregation by stabilizing the functional, monomeric state of MD-2 (9, 27). Therefore, we re-examined the reactivity of select MD-2 mutants with [^{3}H]LOS·sCD14 when these mutants were coexpressed and secreted with the TLR4 ectodomain. Hydrophobic mutants of MD-2 that retained at least partial functional activity with TLR4 (*e.g.* V82F, M85W; see above) reacted with [^{3}H]LOS·sCD14 to form a $M_r \sim$ 190,000 complex representing ([^{3}H]LOS·MD-2·TLR4$_{ecd}$)$_2$ when coexpressed with TLR4$_{ecd}$ (Fig. 4, *A* and *B*). In contrast, more polar mutants (*e.g.* M85K, V82E) did not form a $M_r \sim$ 190,000 complex when coexpressed with TLR4$_{ecd}$ and incubated with [^{3}H]LOS·sCD14 (Fig. 4, *C* and *D*). These findings support the conclusion that the altered properties of the non-reactive MD-2 mutants are due to reduced function and not due to reduced stability/solubility of these mutant proteins.

Identification of Hydrophobic Residues in the TLR4 Ectodomain That Affect TLR4 Activation—It has been proposed that LPS-triggered TLR4 activation depends on agonist-induced interactions between two TLR4·MD-2·LPS ternary complexes involving a region of the TLR4 ectodomain within the central domain (13, 28). We analyzed the crystal structure of the ectodomain of human TLR4, in search of sites that could represent the secondary binding site of the activated complex. We focused on conserved hydrophobic and cationic residues that could be involved in recognition of, respectively, lipid A acyl groups and phosphate and/or ketodeoxyoctonoate groups of LPS that are incompletely coordinated by MD-2. We identified

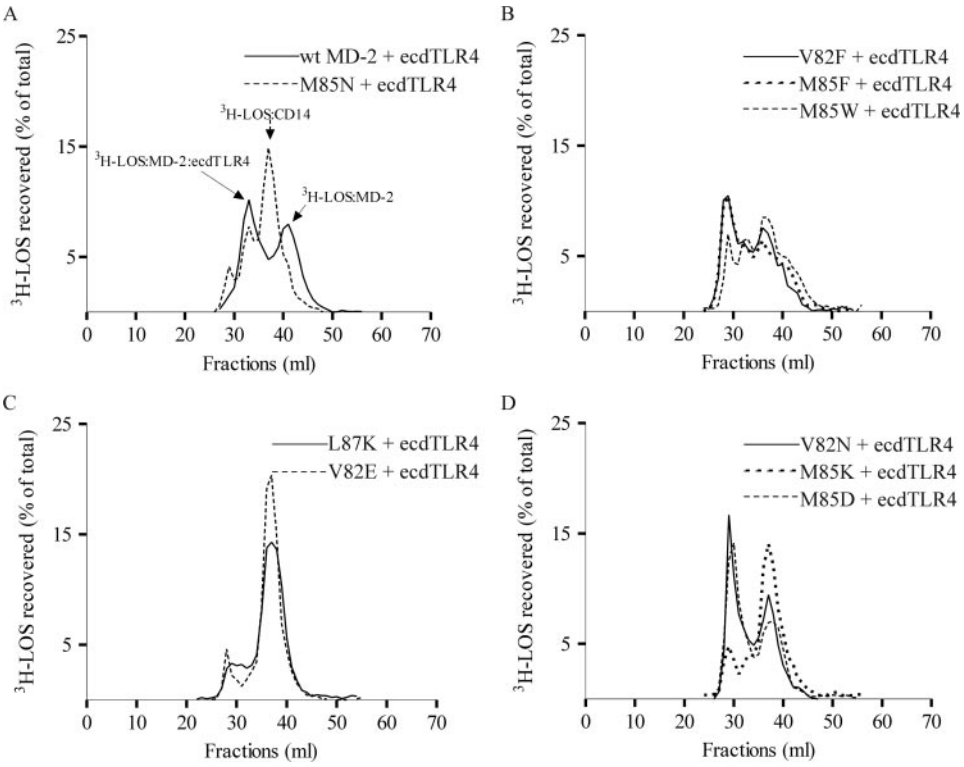

FIGURE 4. **Polar and charged mutants of residues 82, 85, and/or 87 of MD-2 do not react with [^{3}H]LOS·sCD14 to form M_r ~ 190,000 complex when coexpressed with the TLR4 ectodomain.** See the legend to Fig. 3. Experimental conditions and analyses were the same except that sMD-2 was coexpressed with $TLR4_{ecd}$. The M_r ~ 190,000 complex represents the product of transfer of [^{3}H]LOS from [^{3}H]LOS·sCD14 to MD-2·$TLR4_{ecd}$ and elutes at 33 ml. Total recovery of [^{3}H]LOS was typically >70%. The results shown are from one experiment, representative of at least two independent determinations.

residues Phe-440, Phe-463, and Leu-444 as the most promising candidates for hydrophobic interactions. Those residues are conserved in TLR4 orthologs across other species. Replacement of Phe-440 or Phe-463 with alanine abolished LPS signaling (Fig. 5*A*). Replacement of Phe-440 with another bulky, hydrophobic residue, tryptophan, in contrast, substantially preserved TLR4 signaling activity (Fig. 5*B*). Substitution of neighboring Leu-419 on leucine-rich repeat 15 with alanine, in contrast, did not affect LPS responsiveness. However, replacement of Leu-444 on leucine-rich repeat 16 with alanine actually increased cell activation by LPS. These differences in activation were not a consequence of varied expression, as all mutants were expressed at the cell surface in comparable amounts (Fig. 5*E*). Charge reversal mutations of cationic residues within the same region (*i.e.* K388E and K435E) had little or no affect on cell activation by either lipid A or LPS (Fig. 5, *C* and *D*), suggesting that these residues have no major functional roles in lipid A or LPS recognition and TLR4 activation.

DISCUSSION

TLR4 is the most extensively studied of the vertebrate Toll-like receptors. It employs the most complex ligand recognition mechanism with several proteins, such as LPS-binding protein and CD14, required for the extraction and transfer of monomeric LPS from endotoxin aggregates and the Gram-negative bacterial outer membrane to sMD-2 and MD-2·TLR4. Extensive mutagenesis of MD-2, including point mutations, have indicated that several regions of MD-2 are involved in LPS recognition and TLR4 activation (25, 26, 28–32). In this report we identified solvent-exposed hydrophobic residues of MD-2 that are required for binding of agonistic lipid A and receptor activation but, based on earlier crystal structure analyses, do not directly interact with underacylated lipid A analogues that act as TLR4 antagonists. Replacement of Val-82, Met-85, and Leu-87 with other hydrophobic residues preserved the ability of MD-2 to bind hexaacylated LPS/LOS and support TLR4 activation. In contrast, replacement of these hydrophobic amino acids with polar residues led to a marked reduction of receptor activation and, in parallel, reduced transfer of LPS/LOS from CD14 to MD-2. The functional effects of these mutations in MD-2 were manifested when sMD-2 was expressed and secreted with or without TLR4 (ectodomain), strongly suggesting that hydrophobic amino acids at residues 82, 85, and 87 play an important role in the interaction of MD-2 with hexaacylated endotoxin complexed with CD14. Reduced activity of MD-2 mutants that contain the more bulky side chains of aromatic amino acids (*e.g.* V82F, M85F(W); Fig. 2, *B* and *F*) paralleled reduced formation of monomeric LOS·MD-2 complex and increased accumulation of larger [^{3}H]LOS-containing aggregates (Fig. 3), possibly reflecting an increased propensity of these MD-2 variants to engage in MD-2-MD-2 interactions that do not support TLR4 activation.

Biochemical and functional studies of the TLR4 ectodomain have focused on 1) sites within the N-terminal domain needed for agonist-independent interactions with MD-2 and assembly of resting MD-2·TLR4 heterodimers (33, 34), 2) a hypervariable region contributing to species-specific structural requirements for agonists of murine and human TLR4 (13), and most recently, 3) sites within the central domain purportedly mediating agonist-dependent interactions between two TLR4·MD-2·LPS complexes (28). Our findings reveal key roles of Phe-440 and Phe-463 within leucine-rich repeats 16 and 17 of TLR4 in TLR4 activation by hexaacylated LPS. A single mutation (F440A, F463A) renders TLR4 unresponsive to LPS and essentially inert when coexpressed with wt TLR4 (data not shown). The latter finding seems most compatible with an essential role of Phe-440 and -463 in each TLR4 ectodomain in mediating interactions necessary for TLR4 activation. Charged interactions between phosphate groups of lipid A and cationic residues of TLR4 do not seem to play a major role, as charge reversal of either Lys-388 or Lys-435 did not decrease activation.

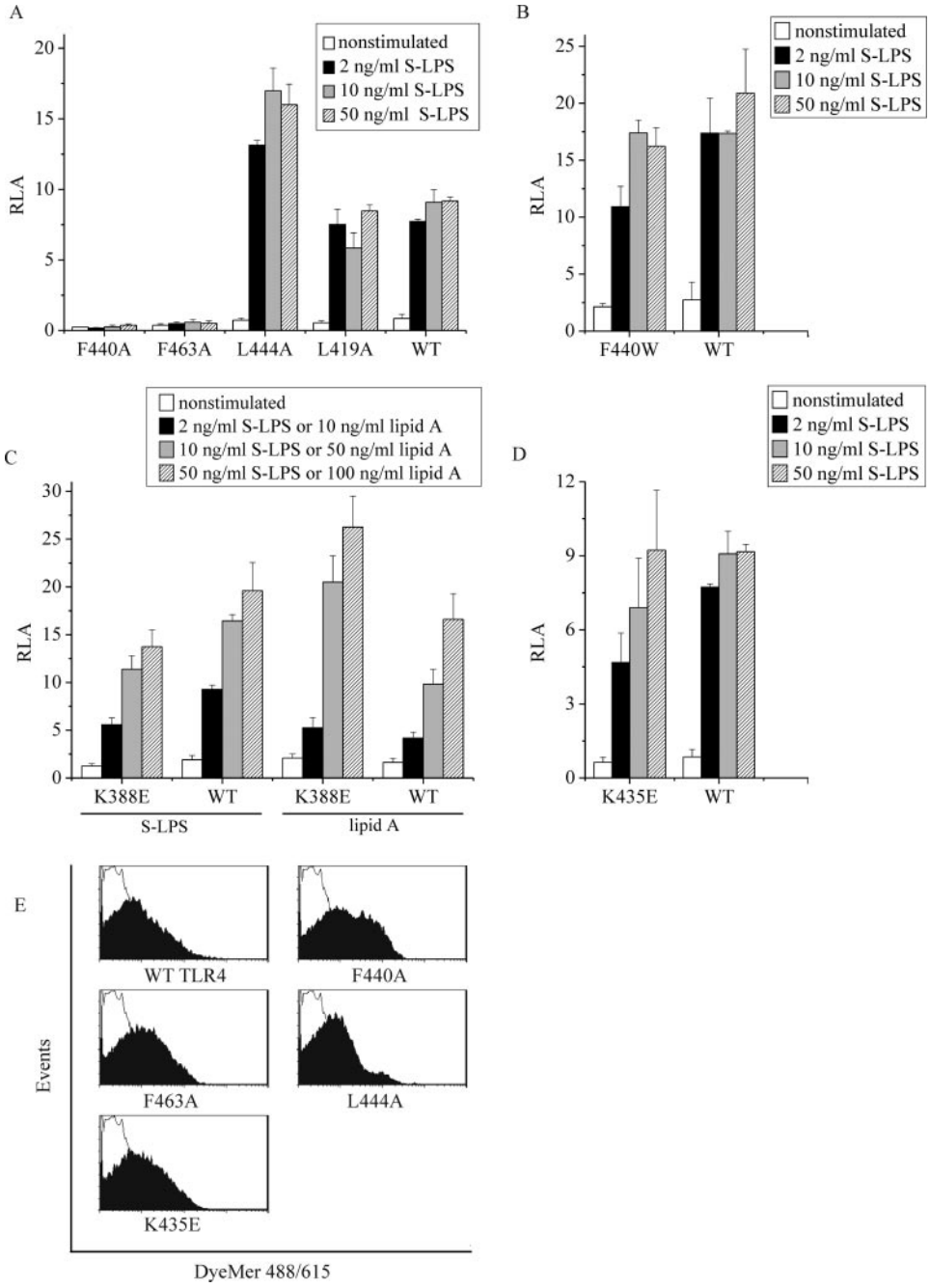

FIGURE 5. **Identification of hydrophobic residues in the central domain of the TLR4 ectodomain that are required for cell activation by LPS.** *A*, solvent-exposed hydrophobic residues of the leucine-rich repeat 16 and 17 of the TLR4 ectodomain were replaced with alanine, and activation was measured by the dual luciferase assay of HEK293 cells transiently transfected with expression plasmids encoding wt or mutant TLR4, wt MD-2, and luciferase reporter plasmids. *B*, effect of replacement of Phe-440 with tryptophan on cell activation by LPS. *C* and *D*, effect of charge reversal mutations of the cationic residues Lys-388 and Lys-435 of the TLR4 ectodomain on cell activation by LPS or lipid A. *E*, surface expression of wt and mutant TLR4 in transiently transfected HEK293 cells was measured by flow cytometry as described under "Materials and Methods." The results shown (*A–D*) represent the mean ± S.D. of triplicate determinations. Each experiment shown is representative of at least three independent experiments.

On the basis of these functional studies, we propose a model of TLR4 activation in which hydrophobic residues of both MD-2 (residues 82, 85, 87, and Phe-126) and TLR4 (440 and 463) are pivotal in the recognition of hexaacylated lipid A and in hexaacylated lipid A (LPS)-induced intermolecular interactions between interacting TLR4·MD-2·LPS complexes (Fig. 6). Our model predicts that hydrophobic side chains of both TLR4 and MD-2 form a narrow hydrophobic pocket that can accommodate parts of a fatty acid chain of lipid A protruding out of MD-2. In the predicted activated receptor complex, residues Phe-440 and Phe-463 of TLR4 are most important in the ectodomain of the receptor as they interact with both distal parts of the acyl chain of lipid A and hydrophobic side chains Met-85 and Phe-126 of MD-2. Replacement of Leu-444 with alanine led to increased TLR4 activation by LPS (Fig. 5*A*), suggesting that wt TLR4 has not evolved to have the highest possible responsiveness to LPS with this particular arrangement of acyl chains. Lower activation of wild type TLR4 may reflect either an effect of Leu-444 to delocalize the hydrophobic cluster on the TLR4 ectodomain or alter interactions with the acyl chain of the activating lipid A moiety.

A salient feature of our model is that it provides an explanation for the activation of TLR4 by agonistic hexaacylated lipid A and specifically for the role of the 3′-hydroxy fatty acid of lipid A. This fatty acid has been shown to be most important for determining the biological activity of lipid A (35). Lipid A with the 3′-secondary fatty acids chain composed of six carbon units cannot activate cell signaling, whereas lipid A species with shorter acyl chains at other secondary acyl positions remain active (35). This is consistent with interaction of the methylene units of the secondary acyl chain closest to the diglucosamine backbone only with MD-2, whereas the more distal carbon units of this fatty acyl chain bind to the surface binding site created by MD-2 and TLR4 (Fig. 6). This arrangement also explains the high protection of the secondary acyl chain from hydrolysis by acyloxyacylhydrolase in the complex with MD-2 (36). Heptaacylated lipid A is an antagonist of human cells (37). This may be explained by our model, as the binding site created at the interface between MD-2 and TLR4 may not be able to accommodate more than one acyl chain.

We have previously proposed that activation of murine TLR4 by taxol involves hydrophobic interactions between mMD-2

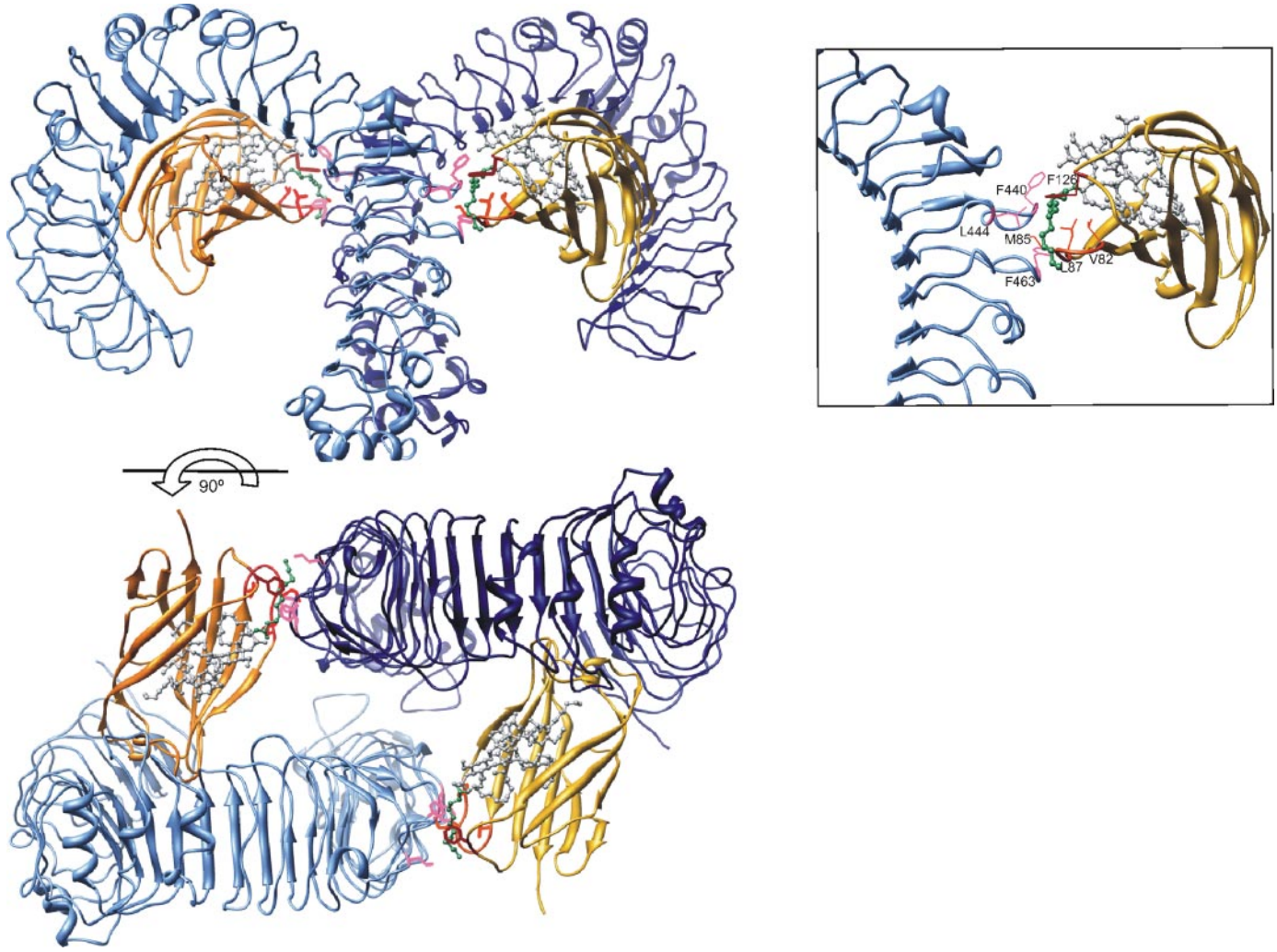

FIGURE 6. **Molecular model of TLR4 activation by LPS; cross-linking of TLR4 ectodomains of two TLR4·MD-2·LPS complexes depends on hydrophobic protein-lipid interactions involving surface hydrophobic residues of MD-2 and TLR4 and a protruding secondary fatty acyl chain of hexaacylated lipid A.** Side chains of amino acid residues that participate in interactions between MD-2 and TLR4 or lipid A are shown as *sticks*. Two perpendicular views of the complex are shown with expanded region of contact of ternary TLR4·MD-2·lipid A complex. See "Discussion" for additional details of the model.

bound taxol and the ectodomain of TLR4 (38), consistent with the model we now present. Our model also suggests that the polymorphic variants D299G and T399I of TLR4, which decrease LPS responsiveness (39), do so in an indirect manner, as neither of these residues in the TLR4 ectodomain appear to directly participate in TLR4·MD-2·LPS and activated receptor complex formation.

Finally, juxtaposition of this model of TLR4 activation with recent molecular models of TLR1/TLR2 activation by triacylated lipopeptides (40) and TLR3 activation by double-stranded RNA (41, 42) suggest striking similarities and dissimilarities. The TLR4 activation complex is symmetric, as is the activation complex of TLR3 and likely that of other homodimeric TLRs. On the other hand the role of a protruding acyl chain of LPS in TLR4 activation resembles the role of the amide bound lipid chain of triacylated lipopeptide that binds into the pocket of TLR1 and triggers formation of TLR1/TLR2 heterodimer (40). The engagement of activating LPS both through the binding pocket of MD-2 and surface hydrophobic residues of MD-2 and TLR4 provides a mechanism that can account for the selective recognition of lipid A as a molecule specific to bacteria by the MD-2·TLR4 complex. It seems likely that this proposed mechanism will also help shed light on the structural basis of bacterial evasion of MD-2·TLR4-mediated recognition, insights that could be translated to more effective LPS antagonists with potent therapeutic potential.

During review of our manuscript, a crystal structure of the TLR4·MD-2·RaLPS complex was published (43). This structure confirms the main features of the proposed activating complex, particularly the participation of a protruding acyl chain of hexaacylated lipid A in the interaction MD-2·LPS with a secondary binding site on the TLR4 ectodomain and is consistent with the key roles of hydrophobic residues of MD-2 (82, 85, 87, 126) and TLR4 (440, 463) that we demonstrated in this work by mutagenesis. Other findings of ours, however, are not fully consistent with speculations made by Park *et al.* (43). In particular, the near normal or even greater than wild-type activity of TLR4 K388E and L444A mutants, respectively (Fig. 5), suggest less important roles of electrostatic interactions involving Lys-388 or hydrophobic interactions involving Leu-444 than proposed by Park *et al.* (43). The most significant difference between our model and the crystal structure is the rotation of the phospho-

rylated disaccharide lipid A backbone by 180°, resulting in exposure of the R2 acyl chain in the crystal structure as opposed to the secondary acyl chain (R3′′) proposed in this paper. Based on our model, we propose that lipid A, as a pseudosymmetric molecule, can fit into the hydrophobic pocket of MD-2 in two orientations, depending on lipid A acyl chain size and arrangement, and perhaps also on substitutions of the phosphate groups. In the crystal structure observed it is possible that the metal ion in the crystallization buffer affected the geometry of the LPS within the complex, as it extensively coordinates the phosphate group.

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